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HSV-1 nucleocapsid egress mediated by UL31 in association with UL34 is impeded by cellular transmembrane protein 140

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ABSTRACT

During HSV-1 infection, the viral UL31 protein forms a complex with the UL34 protein at the cellular nuclear membrane, where both proteins play important roles in the envelopment of viral nucleocapsids and their egress into the cytoplasm. To characterize the mechanism of HSV-1 nucleocapsid egress, we screened host proteins to identify proteins that interacted with UL31 via yeast two-hybrid analysis. Transmembrane protein 140 (TMEM140), was identified and confirmed to bind to and co-localize with UL31 during viral infection. Further studies indicated that TMEM140 inhibits HSV-1 proliferation through selectively blocking viral nucleocapsid egress during the viral assembly process. The blockage function of TMEM140 is mediated by impeding the formation of the UL31–UL34 complex due to competitive binding to UL31. Collectively, these data suggest the essentiality of the UL31–UL34 interaction in the viral nucleocapsid egress process and provide a new anti-HSV-1 strategy in viral assembly process of nucleocapsid egress.

Introduction

Herpes simplex virus 1 (HSV-1) is a double-stranded DNA virus consisting of a viral DNA genome that is surrounded by a capsid and an envelope that is associated with tegument proteins. The HSV-1 infectious cycle consists of viral component synthesis, that begins with viral genome DNA replication and nucleocapsid assembly in the nucleus. Nucleocapsid assembly is followed by nuclear egress, cytoplasmic capsids then bud into TGN-derived vesicles, where they acquire most of the tegument and envelopes, and the progeny is ultimately released from the infected cells. The production of mature, infective virions requires the step-by-step incorporation of every viral component. Each process involves complicated and precise interactions between viral components and cellular molecules. Viral replication is promoted by virus-encoded proteins that alter cellular structures and metabolism, whereas host cells have developed mechanisms for protection against viral replication through evolution (Roizman and Taddeo, 2007).

HSV-1 nucleocapsids are assembled in the nuclei of infected cells and acquire a primary envelope by budding through the inner nuclear membrane into the perinuclear space after the viral DNA genome is encapsidated (Mettenleiter, 2002; Johnson and Baines, 2011). Two conserved viral proteins, which are encoded by the UL31 and UL34 genes, are involved in this biological event, during which they form a complex termed the nuclear egress complex (NEC). The absence of either molecule prevents HSV-1 nucleocapsid egress from the nucleus (Chang et al., 1997; Roller et al., 2000; Reynolds et al., 2001). The UL31 protein has been shown to be a phosphoprotein that is recruited to the nuclear membrane by UL34, which is an integral membrane protein with an N-terminal nucleoplasmic domain and a C-terminal transmembrane domain, to form a complex that co-localizes at the nuclear rim of infected cells (Yamauchi et al., 2001; Zhu et al., 1999; Ye and Roizman, 2000). It has been reported that the interaction between UL31 and UL34 at the nuclear rim and likely, in the nucleoplasm are required for viral nucleocapsid egress from the nucleus during primary envelopment (Reynolds et al., 2001; Zhu et al., 1999; Reynolds et al., 2002; Fuchs et al., 2002; Chang and Roizman, 1993; Kato et al., 2006). By associating with the nucleus and the nuclear lamina network, the UL31–UL34 complex is presumed to alter the nucleus, and nuclear membrane structures and the constructed nucleocapsids subsequently migrate to the inner nuclear membrane for egress and primary envelopment (Reynolds et al., 2004; Simpson-Holley et al., 2004; Simpson-Holley et al., 2005). In addition to the UL31–UL34 complex, the viral protein kinases

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UL13 and US3 as well as cellular protein kinase C (PKC) in the NEC are recruited for viral nucleocapsid egress and envelopment (Reynolds et al., 2002; Kato et al., 2006; Ryckman and Roller, 2004; Mou et al., 2009; Leach and Roller, 2010). These actions suggest that the UL31–UL34 complex likely interacts with the associated host-cell proteins in the process of facilitating viral nucleocapsid egress.

To investigate the details of the mechanism of HSV-1 nucleocapsid egress from the nucleus and of the interaction between the UL31 and UL34 complex and the host cell during viral egress and primary envelopment, we screened host proteins to identify proteins that are capable of interacting with UL31 in cells using yeast two-hybrid analysis. Transmembrane protein 140 (TMEM140), which has an unknown function, was identified and confirmed to interact and co-localize with UL31 in the nucleus upon transfection with UL31 or viral infection. Subsequent studies demonstrated that TMEM140 inhibits HSV-1 proliferation after its over-expression by selectively impeding viral nucleocapsid egress from the nucleus rather than by being exploited by the virion. This inhibitory function is achieved by inhibiting the formation of the UL31–UL34 complex due to the competitive binding of TMEM140 to UL31. This finding demonstrates that the interaction between UL31 and UL34 is essential for viral nucleocapsid egress and that inhibition of the formation of the UL31–UL34 nuclear egress complex by the competitive binding of TMEM140 leads to reduced virion egress, which inhibits viral proliferation. This observation provides new directions for anti-HSV-1 strategies through impeding viral nucleocapsid egress.

Results

Identification of TMEM140 as a protein that interacts with UL31

A yeast two-hybrid screen of the cellular proteins encoded by a cDNA library of human liver tissue was used to identify proteins that interact with the UL31 protein. This assay showed that several host cellular proteins could equally interact with UL31 (Table S1). Based on the demonstration in the literature that UL31 is localized to the nuclear membrane, the potential interactions between three membrane protein molecules, transmembrane 140, transmembrane protein 176 and lysosomal-associated membrane protein 3, and UL31 were further screened in transfected yeast cells. The results revealed an even more clear interaction between TMEM140 and UL31 in yeast cells than what was confirmed using β-gal activity detection (Fig. 1a). Based on these data, the interaction between TMEM140 and UL31 was further assayed using immunoprecipitation in Vero cells. Using an anti-TMEM140 antibody, TMEM140 was detected in the complex that was precipitated by the anti-UL31 antibody using immunoblotting of cells transfected with the TMEM140 and UL31 genes and cells infected with HSV-1 (Fig. 1b). As a gene founded by DNA sequence and annotated in 2003, all we know about TMEM140 is that it is located on human chromosome 7 and predicted to have 4 potential transmembrane domains by bioinformatics analysis (Scherer et al., 2003; Ota et al., 2004). Thus, we performed fluorescence detection for the cellular localization of TMEM140 using an expression plasmid containing a fused EGFP-TMEM140 gene and confirmed the results using immunofluorescence with an antibody specific for TMEM140 in cells. The results showed that TMEM140 was located in the cytoplasm of Vero cells, with low expression and a diffuse or punctuate cytoplasmic staining pattern (Fig. 1c). After transfection of cells with the EGFP–UL31 and Flag-TMEM140 plasmid, soluble TMEM140 was recruited by the expressed UL31 (EGFP–UL31) in the cytoplasm and transferred to the nucleus with it. The distinctive co-localization of both molecules was observed at the nuclear membrane and near the nucleoplasm (Fig. 1d). This co-localization was confirmed in Vero cells infected with HSV-1 (MOI=0.1) (Fig. 1e). Together, these results suggest that UL31 interacts with TMEM140 in HSV-1 infected cells.

TMEM140 inhibits viral proliferation

The identified interaction between TMEM140 and UL31 and the co-localization of the two molecules suggest that TMEM140 has an essential function in viral infection. To study the effects of TMEM140 on HSV-1 infection, we first determined the efficacy of HSV-1 replication and proliferation when the normal expression level of TMEM140 was altered. To ensure the accuracy of this experiment, a MTT assay of the Vero cells transfected with the pcDNA3-TMEM140 expression vector and a siRNA specifically for the TMEM140 gene was performed but did not affect cell viability (Fig. S1). Then, the up-regulation and down-regulation of TMEM140 expression in Vero cells were confirmed using qRT-PCR and western blot assays (Figs. 2a and b, right panel). Compared with the titers in the control infection group, the average virus titers of up-regulation group were reduced at 24–48 hpi (Fig. 2a), indicating that the transient over-expression of TMEM140 inhibits viral proliferation. Moreover, when TMEM140 expression was down-regulated in cells using RNAi (Fig. 2b), virus proliferation was greater than that of the normal control group at 24 and 36 hpi. (Fig. 2b). In the subsequent experiment, re-introduction of the TMEM140 gene into TMEM140 gene knockdown cells overcame the siRNA interference effect on viral replication (Fig. 2c). These results suggest that TMEM140 has a potential inhibitory function in viral proliferation during viral infection.

TMEM140 reduces the rate of HSV-1 nucleocapsid egress

Based on the interaction between TMEM140 and UL31 and the essential function of the UL31 protein in nucleocapsid egress, it was rational to hypothesize that TMEM140 may block the viral nucleocapsid egress process and subsequently lead to a reduction in the viral proliferation rate in cells via its potential interaction with UL31. Therefore, viral nucleocapsid egress during transient over-expression of TMEM140 in HSV-1-infected cells was observed and quantified for 50 visible cells using transmission electron microscopy. The results showed the clear presence of a large number of viral nucleocapsids in the nuclei of infected cells in which TMEM140 was transiently over-expressed (Fig. 3a). In contrast, in the HSV-1-infected control group, fewer viral nucleocapsids were observed in the nucleus, and the majority of nucleocapsids were released into the cytoplasm (Fig. 3a). Statistical analysis showed that approximately 80% of the virions in transiently over-expressing TMEM140 cells were viral nucleocapsids in the infected cell nucleus, compared with 30% for the infected control cells, which meant that only 20% of the virions in the transiently over-expressing TMEM140 cells were released into the cytoplasm, compared with 70% for the infected control cells (Fig. 3b). Therefore, it can be concluded that TMEM140 most likely plays a major role in reducing the rate of viral nucleocapsid egress from the nucleus, subsequently resulting in a reduction in viral proliferation during HSV-1 infection.

TMEM140 blocks the formation of a functional UL31–UL34 complex

It has been reported that the egress of the HSV-1 nucleocapsid is selectively regulated by the virally encoded proteins UL31 and UL34, which interact and bind with each other to form a functional protein complex that co-localizes to the nuclear rim and directly aids in nucleocapsid egress from the nucleus (Reynolds et al., 2001; Reynolds et al., 2002; Fuchs et al., 2002; Simpson-Holley et

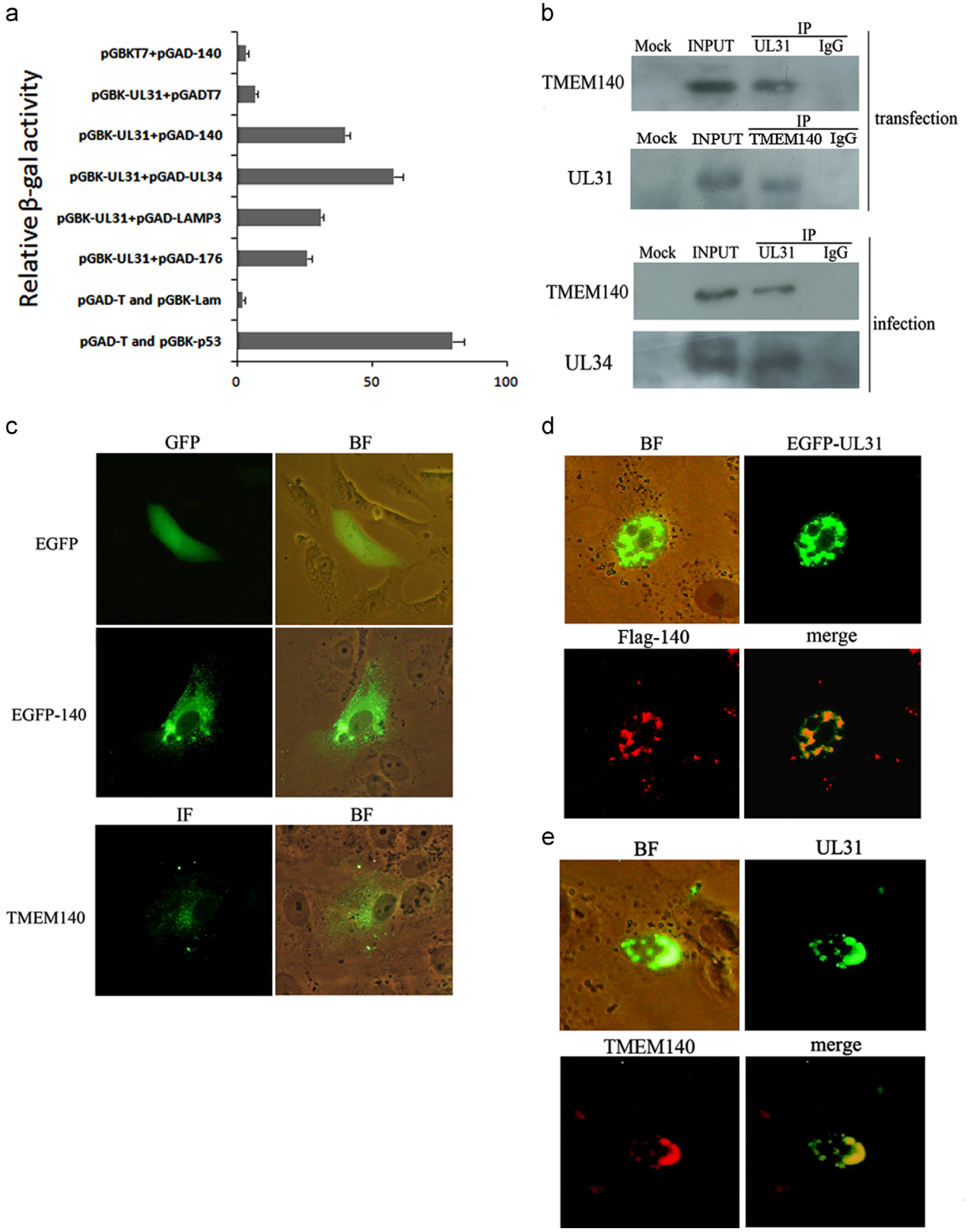

Fig. 1. Interaction between UL31 and TMEM140. (a) Yeast two-hybrid analysis of the physical interaction between UL31 and TMEM140. The yeast strain AH109 is transformed with the indicated plasmid pairs: pGBKT7 and pGAD-140, pGBK–UL31 and pGADT7, pGAD-140 and pGBK–UL31, pGBK–UL31 and pGAD–UL34, pGBK–UL31 and pGAD–LAMP3 or pGBK–UL31 and pGAD-176 or the negative control (pGAD-T and pGBK-Lam) or positive control (pGAD-T and pGBK-p53). The activity of β-galactosidase is measured. The data represent the β-galactosidase activity values relative to those of the negative control from three independent experiments. (b) Co-immunoprecipitation analysis of the interaction between UL31 and TMEM140. Vero cells are co-transfected with pcDNA–UL31 and pcDNA-140 or infected with HSV-1 (MOI=0.1) for 36–40 h. In each case, the cell lysates were firstly immunoprecipitated using mouse anti-UL31 or rabbit anti-TMEM140 (mouse or rabbit IgG as a control) antibody and immunoblotted using a polyclonal TMEM140, UL31 or UL34 antibody. (c) Fluorescence detection of TMEM140 in Vero cells. Vero cells are transfected with pEGFP-140 or pEGFP–N2, as a control, for 48 h, and samples are collected for fluorescence microscopy (upper). Vero cells were fixed and processed for immunofluorescence using the anti-TMEM140 polyclonal antibody (lower). (d, e) The co-localization of UL31 and TMEM140. Vero cells are transfected with pEGFP–UL31 and pFlag-140 for 48 h, and the cells are fixed and processed for immunofluorescence with the anti-Flag monoclonal antibody (d). Vero cells are infected with HSV-1 at a MOI of 0.1, and 20 h post-infection, the cells are fixed and processed for immunofluorescence with the polyclonal anti-UL31 and anti-TMEM140 antibodies (e). Fluorescence image resolution, 20 μm.

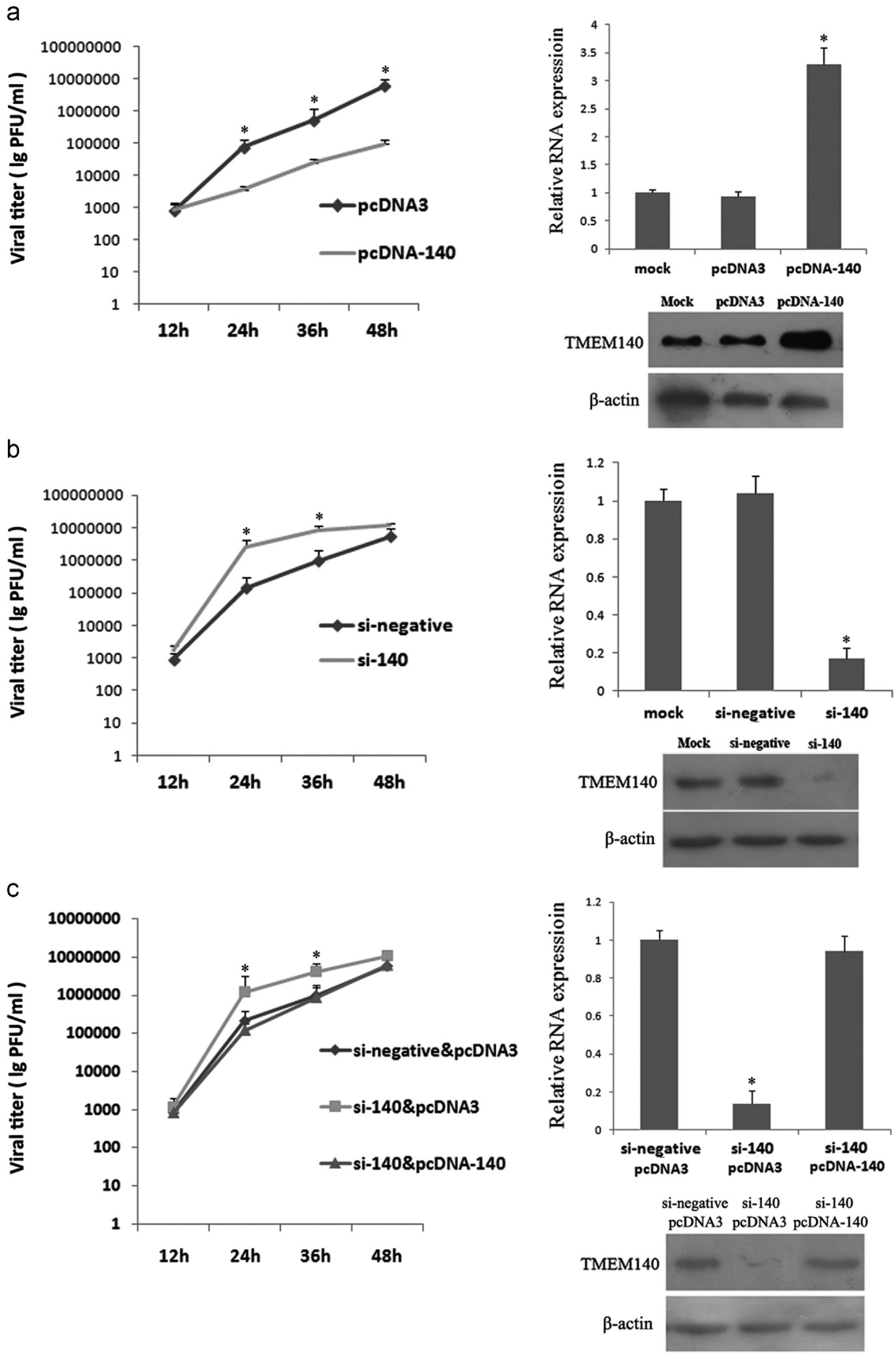

Fig. 2. TMEM140 represses the proliferation of HSV-1. (a) Vero cells in a 6-well plate are transfected with 3 μg of pcDNA-140 or the control pcDNA3 plasmid for 20 h and then infected with HSV-1 (MOI = 0.1). At the indicated times post-infection, samples of infected cells are collected to measure the virus titer using a PFU assay (left). The expression of TMEM140 in the Vero cells is determined using quantitative RT-PCR and immunoblotting (right). (b) Vero cells are transfected with TMEM140 si-140 siRNA or the scrambled interfering RNA as a negative control at a concentration of 150 nM, and the inhibition of TMEM140 expression was quantified using quantitative RT-PCR and immunoblotting (right). At 24 h post-transfection, the cells are infected with HSV-1 (MOI = 0.1), and at the indicated times post-infection, samples of infected cells are collected to measure the virus titer using a PFU assay (left). (c) Vero cells are transfected with 150 nM TMEM140 siRNA for 12 h (scrambled interfering RNA as a negative control) and then transfected with 2 μg TMEM140 expression plasmid pcDNA-140 (pcDNA3 plasmid as a negative control) as indicated. At 24 h post-transfection, the cells are infected with HSV-1 (MOI = 0.1), and at the indicated times post-infection, samples of infected cells are collected to measure the virus titer using a PFU assay (left). The TMEM140 expression level of each group is quantified using quantitative RT-PCR and immunoblotting (right). Error bars represent the standard deviation from triplicate samples. $^{*}P < 0.05$ by Student's *t*-test.

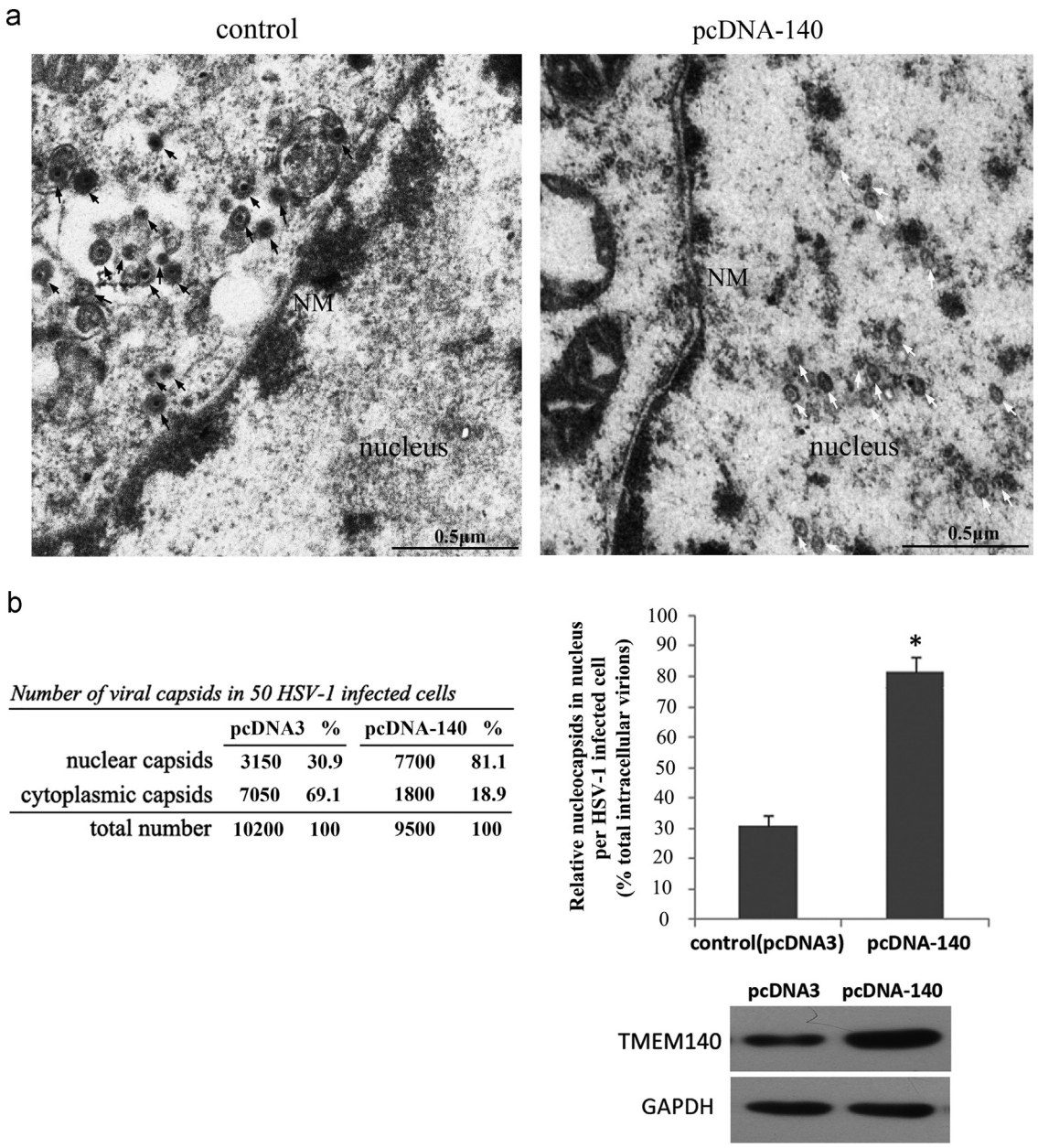

Fig. 3. TMEM140 blocks the nucleocapsid egress of HSV-1. Vero cells transfected with pcDNA-140 or the control pcDNA3 plasmid for 30 h are infected at a MOI of 5 with HSV-1 and incubated at 37 °C. At 17 h post-infection, samples of infected cells were harvested and fixed, and the egress of viral nucleocapsid was assessed using transmission electron microscopy (Hitachi H7650). Virions released into the cytoplasm are indicated by black arrows, and viral nucleocapsids in the nucleus are indicated by white arrows (a). (b) Calculation of the virions per HSV-1-infected cells. The number of viral nucleocapsids as well as cytoplasmic capsids from 50 HSV-1 infected cells are calculated relative to the total virions of the whole cells. Error bars represent the standard deviation from triplicate experiments. The expression levels of the TMEM140 and β-actin (control) proteins are confirmed using western blotting. $^{*}P < 0.05$ by Student's *t*-test (NM, nuclear membrane).

al., 2004). The interaction between TMEM140 and UL31 might interfere with the formation of the UL31–UL34 complex that is required for the viral nucleocapsid egress and maturation of virus particles, possibly resulting in a reduction in viral proliferation. To explore the role of this process, immunoprecipitation was used to observe the interaction between UL31 and UL34 in HSV-1-infected cells in which TMEM140 was transiently over-expressed. As indicated by the immunoprecipitation assay with an antibody against UL31 and the following immunoblotting with an antibody against UL34, the binding between UL31 and UL34 in HSV-1-infected cells was reduced upon the transient over-expression of TMEM140 (Fig. 4a). The immunofluorescence assay using antibodies against UL31, UL34 and TMEM140 indicated that the interaction between UL31 and TMEM140 interfered with the co-localization of UL31 and UL34 to the nuclear rim in cells transfected with the TMEM140 gene and infected with HSV-1, as shown by comparison with the co-localization of UL31 and UL34 in infected control cells (Fig. 4b). The observation for the subsequent immunofluorescence assay and Western blotting using an antibody against the viral capsid structural protein VP5 showed fewer viral nucleocapsids released into the cytoplasm of cells over-expressing TMEM140 than that of the infection control (Fig. 4c). This finding suggested that TMEM140 functioned by interfering with release of the viral nucleocapsid from the nucleus into the cytoplasm through interacting with UL31. In addition, the release of nucleocapsids from the nucleus into the cytoplasm was reduced in cells in which UL31 was down-regulated by a specific UL31-interfering RNA (Fig. 4c, Fig. S2). This observation was further

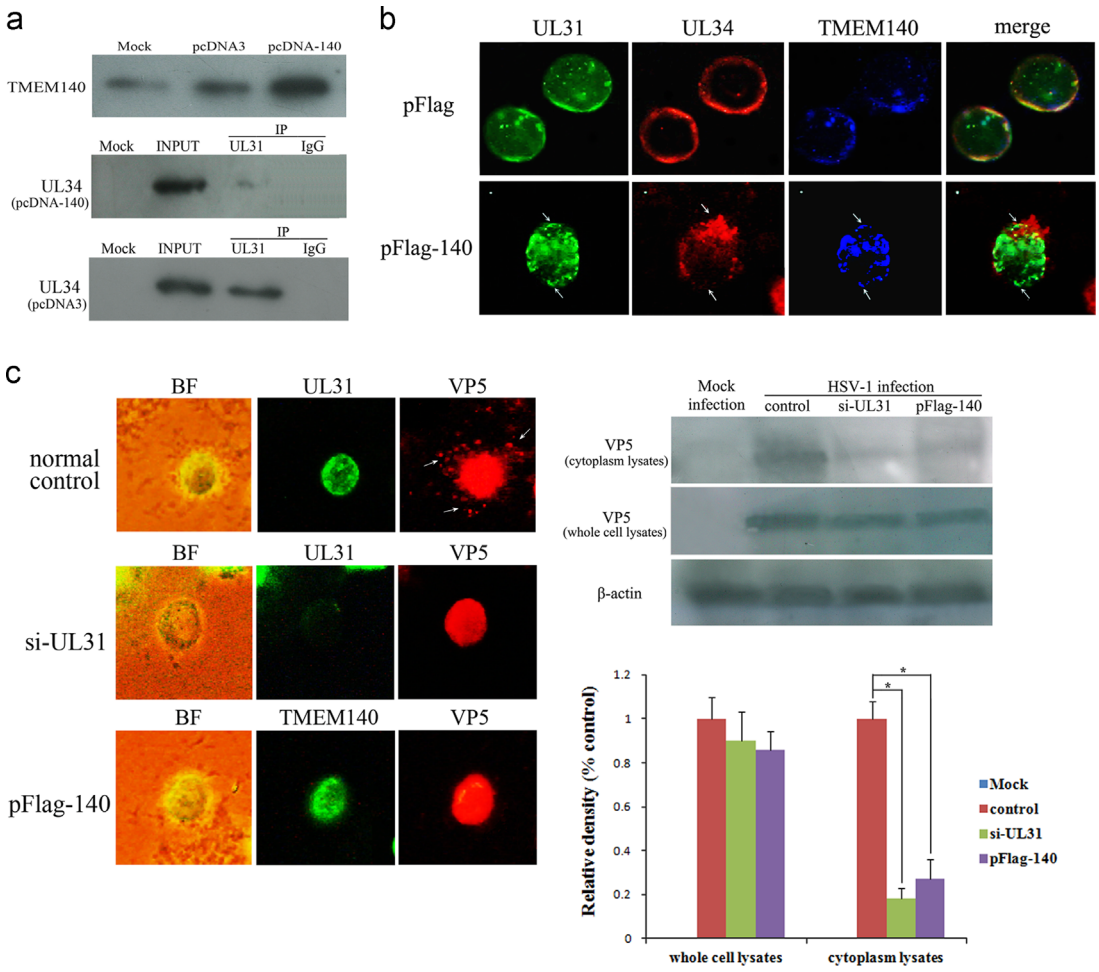

Fig. 4. TMEM140 blocks the interaction between UL31 and UL34 and impedes viral nucleocapsid egress. (a) Interaction between UL31 and UL34 when the expression of TMEM140 is up-regulated. Vero cells in 10-cm dishes were transfected with 6 μg of pcDNA-140 or the control plasmid, pcDNA3, for 30 h and then infected with HSV-1 (MOI=0.1). At 24 h post-infection, equal amounts of whole-cell lysates are immunoblotted with polyclonal TMEM140 antibodies (upper panel) or immunoprecipitated with the mouse anti-UL31 polyclonal antibody or mouse IgG, as a control, and then immunoblotted with the UL34 polyclonal antibody (lower panel). (b) The co-localization of UL31 and UL34 when the expression of TMEM140 was upregulated. Vero cells in 3.5-cm dishes are transfected with 2 μg of pFlag-140 or the control plasmid, pFlag, for 30 h and then infected with HSV-1 (MOI=0.1). At 20 h post-infection, the cells are fixed and processed for immunofluorescence using the polyclonal anti-UL31, anti-UL34 and anti-TMEM140 antibodies. The fluorescent secondary antibodies included FITC-labeled anti-mouse IgG (green), TRITC-labeled anti-mouse IgG (red) and Alexa Fluor 555-labeled anti-mouse IgG (blue). (c) Fluorescence and western blot detection of the inhibition of viral nucleocapsid egress by TMEM140. Vero cells are transfected with 100 nM UL31 si-UL31 siRNA (or the scrambled interfering RNA si-negative as a control) or 3 μg of pFlag-140 plasmid for 30 h and then infected with HSV-1 (MOI=0.1). At 18 h post-infection, the cells are fixed and processed for immunofluorescence using the anti-VP5, anti-Flag and anti-UL31 antibodies. The fluorescent secondary antibodies included FITC-labeled anti-mouse IgG (green) and TRITC-labeled anti-mouse IgG (red). Moreover, the cell lysates of whole cells or cytoplasm are collected, and equal amounts of the protein supernatants are subjected to western blotting using mouse anti-VP5 and anti-β-actin (control) monoclonal antibodies. The histogram represents the mean ± SD of the densitometric scans for the protein bands from three experiments that are normalized against β-actin and expressed as a percentage of the control group. Fluorescence image resolution, 20 μm.

supportive for the above suggestion. All of results here lead to a conclusion that the interaction between TMEM140 and UL31 interferes with the formation of the UL31–UL34 complex and might impact viral proliferation.

TMEM140 binds to UL31 via the CR1 domain

We lastly identified the domain of UL31 that interacts with TMEM140. Because previous studies have indicated that the critical binding site between the UL31 and UL34 proteins is the first conserved region (CR1) of UL31 (Lotzerich et al., 2006; Schnee et al., 2006), an expression vector, pCMV-HA-31CR1, encoding the CR1 sequence was constructed and co-transfected with the TMEM140 expression vector into cells. The immunoprecipitation results indicated a clear interaction between the TMEM140 and UL31 CR1 proteins (Fig. 5). The site-directed mutation of six conserved amino acids in the CR1 region showed that the critical binding site in CR1 that interacts with TMEM140 includes the 94th residue, which is a leucine, the mutation of which inhibited the interaction between TMEM140 and UL31 (Fig. 5). These results suggest that TMEM140 interacts with UL31 via a critical binding site that is located in the UL31 CR1 region, which is the same binding site as that for the interaction with UL34. This result further supports our primary hypothesis that TMEM140 inhibits HSV-1 proliferation via blocking the formation of the functional UL31–UL34 complex in cells.

Discussion

Previous studies have demonstrated that the replication of HSV-1, which has a complicated proliferation mechanism, involves interactions between multiple virus-encoded molecules and various cellular molecules. Importantly, the interaction between host and virus molecules alters the cellular environment and subsequently leads to the final pathogenic process (Roizman and Taddeo, 2007).

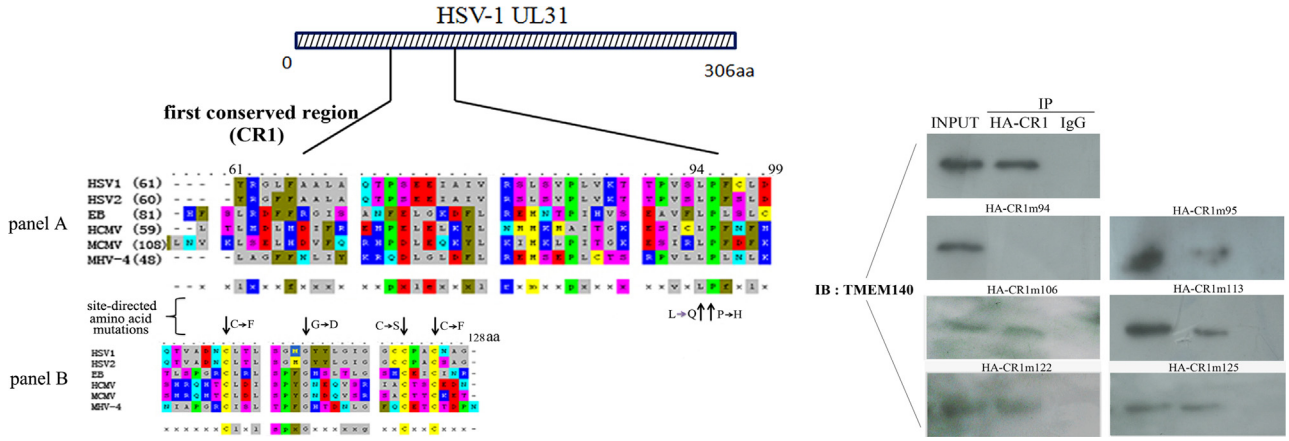

Fig. 5. Interaction between UL31 CR1 and TMEM140. Diagram of the herpes UL31 first conserved region (CR1) and the six site-directed amino acid mutations (left). Vero cells in 10-cm dishes are transfected with the pCMV-HA-31CR1 plasmid, which contains the HSV-1 UL31 CR1 coding region, or the pCMV-HA-31CR1m plasmids, in which the UL31 CR1 contained mutations at amino acids 94, 95, 106, 113, 122 and 125, as indicated. At 48 h post-transfection, the cells are immunoprecipitated using a mouse anti-HA polyclonal antibody or mouse IgG as a control and immunoblotted using the TMEM140 polyclonal antibody (right).

Previous experiments have revealed that the process from nucleocapsid formation to assembly of the final mature virion is a typical biological event that depends on the interaction between virus-associated molecules and cellular components at every step (Mettenleiter, 2002; Johnson and Baines, 2011). It is presumed that the process by which the HSV-1 nucleocapsid in the nucleus passes through the double inner nuclear membrane and buds into the cytoplasm is directly initiated by the formation of the nuclear egress complex (NEC), the majority of which consists of the virus-encoded proteins UL31 and UL34 (Mettenleiter, 2002; Mettenleiter et al., 2013). The interaction between these two molecules is likely followed by the recruitment of various components including cellular protein kinase C and the viral molecules US3 and UL13, as well as the cellular protein nucleolin and the virus-encoded proteins UL12 and UL47, to alter the nuclear membrane structure through phosphorylation of the nuclear lamina network (Johnson and Baines, 2011; Kato et al., 2006; Reynolds et al., 2004; Simpson-Holley et al., 2005; Mou et al., 2009; Leach and Roller, 2010; Sagou et al., 2010; Liu et al., 2014). Thus, the interaction between UL31 and UL34 likely plays an essential role in the formation of progeny virions. The TMEM140 molecule that was identified in the current study as an interacting partner of UL31 was not recruited to facilitate mature virion assembly, but affected viral nucleocapsid egress and proliferation through impeding the UL31–UL34 interaction and, most likely, NEC formation. The results provide substantial evidence indicating that NEC formation, especially the UL31–UL34 interaction, plays a vital role in HSV-1 nucleocapsid egress. Thus, the inhibition of this process will lead to inhibition of the entire infectious process, at least to some extent, following a decline in the number of mature progeny virions.

Based on the MOI (0.1) that was used in this study, we identified the presence of an interaction between TMEM140 and UL31 during the process of viral nucleocapsid egress and mature virion assembly at 18 h – 36 hpi. This time is reasonable because it covers two passages of virus proliferation in cell. Importantly, this interaction was confirmed using immunoprecipitation and cellular fluorescence co-localization assays, and no impact of TMEM140 on the transcription of the viral genome was found (Fig. S3). It is proposed that TMEM140 binds to UL31 in the CR1 structural domain, which is also the domain of UL31 that is bound by UL34, suggesting that TMEM140 affects UL31–UL34 binding in a steric manner. As shown using the immunoprecipitation and cellular fluorescence co-localization assays with HSV-1-infected cells transiently over-expressing TMEM140; TMEM140-UL31 reduced the efficiency of UL31–UL34 binding. This result contributes to our understanding of the reduction in HSV-1 proliferation in cells transiently over-expressing TMEM140 compared with that in normal control cells. The electron microscopy observations and the immunofluorescence analysis of the VP5 structural protein that were performed in HSV-1-infected cells revealed that over-expression of TMEM140 simply resulted in a decrease in the HSV-1 nucleocapsid egress efficiency. This result indicates that the viral proliferation efficiency could be affected by TMEM140.

The potential mechanism by which the HSV-1 proliferation efficiency was reduced was addressed in our current study by the identification of the ability of TMEM140 to impact the efficiency of HSV-1 nucleocapsid egress via binding to the virus-encoded protein UL31; however, the details of how this molecule affects HSV-1 nucleocapsid egress and reduces viral proliferation via the entire NEC complex must be further investigated. HSV-1 nucleocapsid egress via NEC formation is believed to be an important step in viral proliferation, and the UL31 and UL34 proteins have been found to play important roles in viral gene replication and proliferation by recruiting glycoproteins D and M to increase efficacy (Chang et al., 1997; Wills et al., 2009; Roberts and Baines, 2011). Therefore, inhibition of the UL31–UL34 interaction using a mimic molecule, such as TMEM140 or a chemical compound may be a potential strategy to control HSV proliferation in cells.

Experimental procedures

Cell lines, viruses, plasmids and reagents

African monkey kidney cells (Vero) were cultured in DMEM containing 10% calf serum at 37 °C. HSV-1 virus (F strain), which was preserved by our laboratory, was grown in a monolayer of Vero cells.

The eukaryotic expression plasmids pcDNA-UL31, pcDNA-140, pGAD-140, pGBK-UL31, pEGFP-UL31, pEGFP-140, pFlag-140 and pGAD-UL34 were constructed by inserting the HSV-1 UL31, UL34 or cellular TMEM140 gene sequences into the pcDNA3 (Invitrogen), pGBKT7, pGADT7, pEGFP-N2 (Clontech) and p3XFLAG-CMV (Sigma-Aldrich) vectors. The UL31 CR1 region, 151–390 bp, was inserted into the eukaryotic expression vector pCMV-HA (Clontech) to construct the pCMV-HA-31CR1 plasmid. The pCMV-HA-31CR1 mutation plasmids, in which the leucine (L) at position 94 of CR1 was mutated to glutamine (Q), the proline (P) at position 95 was mutated to histidine (H), the cysteine (C) at position 106 was mutated to phenylalanine (F), the glycine (G) at position 113 was

mutated to aspartic acid (D), the cysteine (C) at position 122 was mutated to serine (S), and cysteine (C) at position 125 was mutated to phenylalanine (F) (Fig. 5), were constructed using a Site-directed Gene Mutagenesis Kit (Beyotime). The oligonucleotide sequences for the amplification of the above-mentioned genes are shown in Table S2. All recombinant plasmids were analyzed and confirmed using enzyme digestion and sequencing. The plasmids pGBKT7-Lam, pGBKT7-p53 and pGADT7-T from Clontech were stored in our laboratory.

A rabbit anti-TMEM140 polyclonal antibody (HPA016871) was purchased from Sigma-Aldrich (USA). The mouse anti-HA and anti-Flag monoclonal antibodies (M20003 and M20008) were purchased from Abmart (Shanghai, China). The mouse UL31 and UL34 antisera were produced by our laboratory, and their specificities were identified before use in experiments (Fig. S4). The rabbit anti-GAPDH monoclonal antibody (AG019) and mouse anti-β-actin monoclonal antibody (AA128) were purchased from Beyotime Biotechnology (Wuhan China). The mouse anti-VP5 monoclonal antibody (6F10) was purchased from Santa Cruz Biotechnology. The peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Dingguo Biotechnology (Beijing, China). The FITC-labeled goat anti-mouse IgG (H+L) and Alexa Fluor 555-labeled donkey anti-mouse IgG (H+L) were purchased from Beyotime Biotechnology. The TRITC-labeled goat anti-mouse IgG was purchased from Dingguo Biotechnology (Beijing, China). The Nuclear and Cytoplasmic Protein Extraction Kit (P0027) was purchased from Beyotime Biotechnology.

Yeast two-hybrid screen and β-galactosidase assay

A yeast two-hybrid screen was performed using the MATCHMAKER GAL4 two-hybrid system 3 (Clontech, USA) according to the manufacturer's protocols (Zhang et al., 2011). Briefly, to test the potential interaction between UL31 and TMEM140, the yeast strains AH109 or Y187 were transformed with pGAD–TMEM140 and pGBK–UL31 and plated onto SD/-Leu/-Trp (DDO) or SD/-Ade/-His/-Leu/-Trp medium (QDO). Yeast transformed with pGBKT7 plus pGAD–TMEM140 or pGBK–UL31 plus pGADT7 were used as self-activation controls. Yeast transformed with pGBKT7-P53 plus pGADT7-T or pGBKT7-lam plus pGADT7-T were used as positive and negative controls, respectively. The plates were incubated at 30 °C until the appearance of colonies between 3 and 7 days. Then, Y187 colonies growing on the DDO agar plates were picked and subjected to β-galactosidase activity analysis following the manufacturer's instructions. The data represent the β-galactosidase activity values relative to those of the negative control from three independent experiments.

Fluorescence detection

Vero cells transfected with the chimeric EGFP plasmids (Lipofectamine 2000, Invitrogen) and/or infected with HSV-1 were grown for 24–48 h and subsequently treated with 4% polyformaldehyde and 0.2% Triton X-100 for 10 min to facilitate observation using a fluorescence microscope. Then, the transfected cells were incubated with a mouse anti-UL31 antibody (1:400), anti-UL34 antibody (1:600), anti-TMEM140 antibody (1:50), anti-VP5 antibody (1:500), anti-Flag antibody (1:500) or anti-HA antibody (1:500) for 2 h and further incubated with a FITC-, Alexa Fluor 555-, or TRITC-conjugated goat anti-mouse antibody (1:250) for 1 h. The slides were finally examined using a Nikon E600 fluorescence microscope.

Cell viability assay

A total of 2×10^4 Vero cells were plated onto 96-well plates for 16 h. Each well of cells was then transfected with different plasmids or siRNA. At different post-transfection times (12 h, 24 h, 36 h, 48 h and 60 h), the culture medium was removed and replaced with culture medium containing 20 μl of sterile MTT dye (5 mg/ml). After incubation at 37 °C for 4 h, the MTT solution was removed, and 150 μl dimethyl sulfoxide (DMSO) was added to each well followed by measuring the absorbance at 570 nm on an enzyme immunoassay analyzer (Bio-Rad, USA).

Quantitative RT-PCR

Total RNA was extracted from the transfected Vero cells using TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using the supermoIII RT kit (Bioteke, Beijing). Real-time PCR analysis of the TMEM140, UL31, ICP0, TK, gC and control β-actin mRNAs was performed using the SYBR Premix Ex Tag II (Takara) (Table S3). The TMEM140 and UL31 levels were normalized to the β-actin RNA level, and the expression levels were calculated as $2^{-\triangle\triangle Ct}$. All experiments were repeated three times, and the mean value was calculated for statistical analysis.

Western blot analysis

The proteins from cell lysates were quantitated, and equal amounts of supernatant protein were subjected to 10% SDS-PAGE. The proteins were transferred to a PVDF membrane, which was then blocked with 5% BSA-TTBS (BSA, 5%; Tris–HCl, 100 mM, pH 7.5; NaCl, 0.9%; Tween-20, 0.2%). The membranes were subsequently treated with a specific primary antibody and a goat anti-mouse or anti-rabbit IgG-HRP secondary antibody. Enhanced chemiluminescence (ECL) detection of antigen–antibody complexes using Immobilon Western HRP Substrates (Millipore) was performed according to the standard protocol (Guo et al., 2012). Bands were analyzed using densitometry and the ChemiDoc EQ (Bio-Rad, USA) system. The band intensities were normalized against β-actin and compared with that of the control group, and the relative intensity ratios were calculated. Western blot analyses of the proteins of interest were examined in three separate experiments.

Immunoprecipitation

Vero cells were cultured in 10-cm cell culture plates, and the next day, the cells were co-transfected with the indicated plasmids (Lipofectamine 2000, Invitrogen) or infected with HSV-1 (MOI=0.5). The cells were washed with PBS 36–48 h post-transfection or post-infection and lysed for 30 min in ice-cold RIPA lysis buffer (NaCl, 150 mmol/L; NP-40, 1%; sodium deoxycholate, 0.5%; SDS, 0.1%; Tris–HCl, 50 mM, pH 7.5). The cell lysate was centrifuged at 14,000*g* for 10 min, and the supernatant was precleared by incubation with protein A+G agarose beads (Beyotime Biotechnology) for 1 h at 4 °C. An equal aliquot of the cell lysates was reserved as an input sample, and the remaining lysates were incubated with anti-UL31 or anti-HA antibodies under standard immunoprecipitation conditions and incubated at 4 °C for 3 h with gentle agitation. Normal mouse IgGs were used as controls. Protein A-agarose beads were added to the protein-antibody mixture and incubated overnight at 4 °C with rotation. The beads were collected using centrifugation, washed three times with RIPA buffer and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Viral titration

Vero cells were co-transfected with the indicated plasmids (pcDNA3 as a control) for 20 h (Lipofectamine 2000, Invitrogen) and then infected with HSV-1 at a MOI of 0.1. Then, 12–48 h post-infection, samples of infected cells were collected to measure the virus titer using a PFU assay. Briefly, viral stocks were serially diluted 10 times, and the diluted stocks were added to six-well plates that were coated with Vero cells. Subsequently, pre-warmed 1 × DMEM with 1% agar was added to the cell surface, and the plates were

incubated at 37 °C for 4 to 5 days. Before the quantification of plaque formation, 4% paraformaldehyde and 0.5% crystal violet were added to each well to fix and dye the cells, respectively.

RNA interference (RNAi) for TMEM140

The siRNA duplexes si-140 (targeting TMEM140) and si-UL31 (targeting UL31) and the scrambled interfering RNA, si-negative, which was used as the negative control, were chemically synthesized using the 2'OME modification by GenePharma (Shanghai, China) and RiboBio (Guangzhou, China). (Table S4). All siRNAs were stored in 0.1% diethylpyrocarbonate-treated water at −80 °C. Before the experiment, the transfection efficiency of the siRNA was tested using fluorescence-labeled siRNA. The result showed a transfection efficiency of siRNA that was higher than 70% (Fig. S5). Based upon this identified transfection efficiency, Vero cells were seeded into the wells of 6-well plates. On the day after seeding, the cells were transfected with si-140, si-UL31 or si-negative at a final concentration of 100–150 nM using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. At 40 h after transfection, the transfected cells were harvested, and total cellular protein extracts were prepared for western blot analysis.

Electron microscopy

HSV-1-infected Vero cells were fixed with 2.5% glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7.3) and 1% osmium tetroxide, followed by washing with double-distilled water three times. The sample was subjected to gradient dehydration with 50%, 70%, 95% and 100% ethanol prior to being embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate for transmission electron microscopy observations (Hitachi H7650, Japan).

Statistical analysis

The data that were obtained from all experiments are presented as the mean ± standard deviation (SD), and $P < 0.05$ was considered to be statistically significant using Student's *t*-test.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.06.034.

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